RAPID COMMUNICATION

Glomerular Basement Membrane Discontinuities

Scanning Electron Microscopic Study of Acellular Glomeruli

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Glomerulonephritides which develop necrotizing and crescentic lesions usually have glomerular basement membrane (GBM) disruptions when carefully examined by light microscopy or transmission electron microscopy. Despite numerous excellent and detailed ultrastructural investigations of GBM discontinuities, a complete appreciation of their actual number, appearance, and distribution within a glomerulus has been difficult to achieve by reconstruction of two-dimensional light or transmission electron microscopic images. Selective removal of podocytes by a sequence of lytic and solubilization procedures has been developed which exposes any structural alteration of the GBM to direct examination by scanning electron microscopy. A case of idopathic, immune-complex-negative, focal-segmental necrotizing glomerulonephritis has been studied by this technique, permitting three-dimensional visualization of the GBM defects which result in free communication between the vascular and urinary spaces. These disruptions were distinctive by their frequency within an affected lobule, variable size, and sharply demarcated edges. Application of this technique to human renal biopsies is capable of enhancing our understanding of the morphologic alterations occurring in human glomerulonephritis. (Am J Pathol 1985, 119:357-360)

DISRUPTION of the structural integrity of the glomerular basement membrane (GBM) occurs in diverse forms of glomerulonephritis and represents the most severe form of glomerular injury. Such a breech in the barrier between the vascular and urinary space permits direct extravasation of protein, red cells, and fibrin into Bowman's space, resulting in crescent formation.

Attempts to study GBM discontinuities ultrastructurally have been hampered by their infrequent occurrence in affected glomeruli.[1-7] The use of serial sections has been advocated to increase the yield of GBM gaps; however, appreciation of their appearance and their intraglomerular distribution remains difficult.[3-7]

Direct three-dimensional visualization of structural alterations of the GBM and their distribution within a glomerulus is possible by combining scanning electron microscopy with cell extraction techniques.[8,9] This procedure has been applied to a case of focal segmental necrotizing glomerulonephritis permitting the first three-dimensional observations of GBM disruptions in a case of necrotizing glomerulonephritis.

Materials and Methods

An open renal biopsy was received, and portions were fixed in 10% formalin, 2% glutaraldehyde and frozen in liquid nitrogen for light-microscopic, transmission electron microscopic, and direct immunofluorescence study, respectively. Paraffin-embedded tissue was sectioned at 3 μ, and serial sections were stained with hematoxylin and eosin (H&E), periodic and acid-Schiff, Masson's trichrome and Jones' methenamine silver. Tissue for transmission electron microscopy was rinsed in phosphate buffer (0.1 M, pH 7.2), postfixed in 1% osmium tetroxide, dehydrated in graded alcohols and propylene oxide, and embedded in Polybed 812 (Polysciences). Ultrathin sections were stained with 2% aqueous magnesium uranyl acetate for 30 minutes and than by lead citrate for 5 minutes. A JEOL JEM 100 CX

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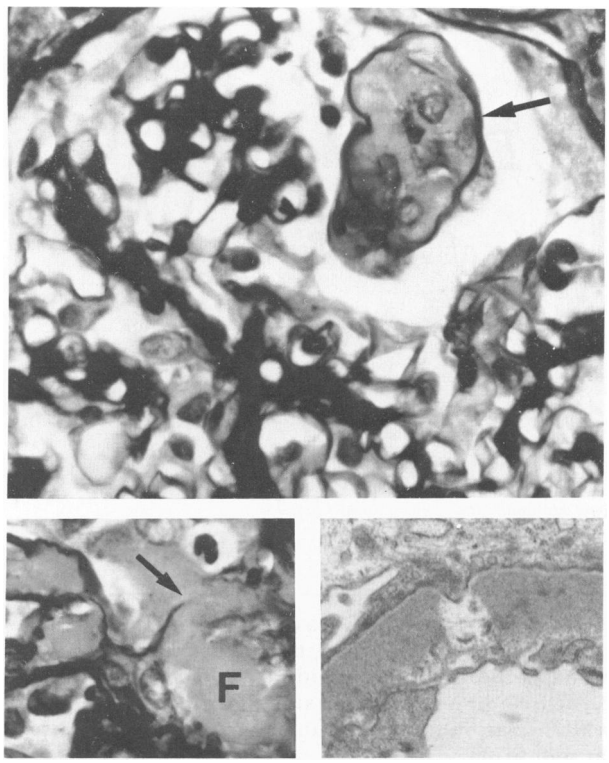

Figure 1—Light micrograph showing segmental intracapillary loop thrombosis (*arrow*). (Jones' methenamine silver, ×425) **Insets—Left**—Loop thrombosis and GBM disruption (*arrow*). *F*, fibrin. (×425) **Right**—Transmission electron micrograph showing an indented lucent GBM focus. (×16,000)

analytical TEMSCAN electron microscope was used at 60 kv. Frozen tissue for immunofluorescence was cryosectioned at 4 μ and reacted with antisera specific for immunoglobulin heavy chains, C3, and fibrin.

Cell extraction employed the frozen tissue from the open renal biopsy after completion of direct immunofluorescence. Control tissue included 10 frozen normal rat kidneys and 5 frozen normal adult kidneys obtained at the time of nephrectomy for renal cell carcinoma. Cell extraction was performed as previously described, by a slight modification of the original method of Carlson and Kenny.[8-10] The modification consisted of using frozen rather than fresh kidneys, reducing the osmotic lysis step from 48 to 24 hours, and increasing the incubations in DNase and deoxycholate.[8,9] Briefly, the schedule employed consisted of sequential incubations in the following solutions: 5 mM EDTA, 4 C, 24 hours; 3% Triton X-100, room temperature, 12 hours; 0.025% deoxyribonuclease in 1 M NaCl, room temperature, 6 hours; and 4% deoxycholate, room temperature, 12 hours. All solutions contained 0.1% sodium azide. The specimens were rinsed in distilled water for 30 minutes between each solution.

Acellular (extracted) tissue was fixed in phosphate-buffered (0.1 M, pH 7.2) 2% glutaraldehyde, washed in buffer, postfixed in 1% osmium tetroxide in phosphate buffer (0.1 M, pH 7.2), and washed in buffer. Dehydration employed graded alcohols and critical-point drying followed by vacuum coating with gold-palladium. A JEOL JEM 100 CX analytical TEMSCAN electron microscope was used at 20 kv accelerating voltage.

Results

Routine Morphologic Studies

The renal biopsy contained roughly 100 glomeruli, none of which contained hypercellularity or sclerosis. Approximately 10% of glomeruli contained segmental capillary loop thrombi and/or capillary loop disruptions with fibrin and red cells in Bowman's space (Figure 1A and left inset). Although a rare segmental cellular crescent was present, most segmental lesions appeared "early," with fibrin and red cell extravasation into Bowman's space but associated with little cellular reaction.

Direct immunofluorescence and transmission electron microscopy each demonstrated fibrin extravasation in Bowman's space but revealed no evidence of immune complex deposition. Transmission electron microscopy showed segmental capillary loop thrombosis with an intact endothelial cell lining, a large disruption of the GBM with fibrin extravasation into Bowman's space, as well as occasional small subepithelial indented lucent or rarified foci in the GBM (Figure 1A, right inset), unassociated with immune complexes or inflammatory cells.

Acellular Scanning Electron Microscopy

Acellular SEM of the patient's biopsy and of control rat and human glomeruli showed complete extraction of the cellular components, exposing the subepithelial aspect of the GBM to direct scanning electron microscopic examination (Figure 2A). The acellular GBM of uninvolved glomeruli in the patient's biopsy was indistinguishable from that of control rat and human glomeruli. Each had a uniform-appearing, smooth subepithelial surface. This appearance is indistinguishable from that reported by Bonsib in studies of minimal change nephrotic syndrome and is in agreement with Carlson and Kenny's observations of normal acellular rat, rabbit, monkey, and human glomeruli.[9,10]

Approximately 10% of the glomeruli from the open biopsy had a segment in which one to several capillary loops contained multiple discrete perforations or holes in the GBM (Figure 2A–C). These perforations were round to oval, varied greatly in size, and tended to clus-

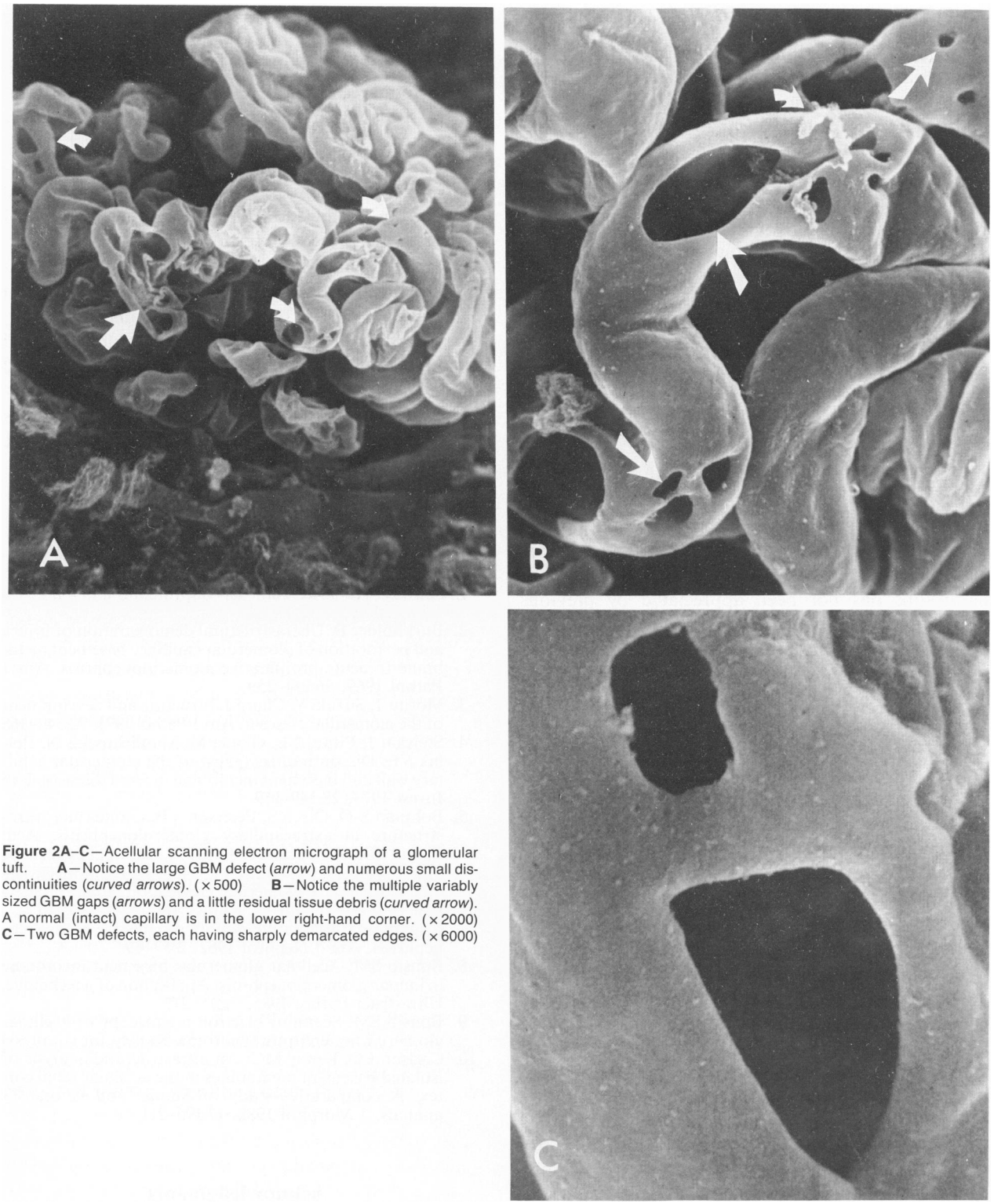

Figure 2A–C—Acellular scanning electron micrograph of a glomerular tuft. **A**—Notice the large GBM defect (*arrow*) and numerous small discontinuities (*curved arrows*). (×500) **B**—Notice the multiple variably sized GBM gaps (*arrows*) and a little residual tissue debris (*curved arrow*). A normal (intact) capillary is in the lower right-hand corner. (×2000) **C**—Two GBM defects, each having sharply demarcated edges. (×6000)

ter within an affected lobule. The edges of the gaps were sharp and tended to curl slightly into the capillary loop. Tiny pinpoint defects were also noted, which did not appear to extend through the entire thickness of the GBM. These may represent the earliest stage in the development of a gap and may correspond to the indented subepithelial lucent foci visualized by transmission electron microscopy (Figure 1A, right inset).

Discussion

Application of acellular scanning electron microscopy to this case of necrotizing glomerulonephritis has permitted three-dimensional visualization of GBM defects which precede crescent formation. The GBM gaps observed are believed to be an accurate portrayal of these lesions as they exist *in vivo*, rather than an artefact of obtaining or handling the tissue. This is supported by finding that the glomerular tuft architecture was intact, with defects noted at various levels within the tuft and on various sides of a loop. This distribution of lesions would be difficult to produce by simple mechanical injury. In addition, in control rat and human glomeruli, in previous reports by the author of human biopsies for nephrotic syndrome and in previous reports by Carlson and Kenny of normal rat, rabbit, monkey, and human glomeruli, similar lesions of the GBM have not been observed.[8-10]

The focal and segmental distribution of the lesions noted by light microscopy was equally apparent by acellular scanning electron microscopy. An unexpected finding, however, was the large number of defects present within an affected lobule. Such a multiplicity of disruptions has not been appreciated by previous transmission electron microscopic studies of GBM discontinuities, even those employing serial sections.[3-7] It would seem that the number, size, and distribution of GBM defects within a glomerular tuft would influence the size of the crescent subsequently formed as well as size of the glomerular scar which develops when the destructive process terminates.

The largest gaps are clearly of sufficient dimensions to allow unfiltered serum and cells to spill directly into Bowman's space, as demonstrated by the pools of fibrin and red cells seen in light microscopic observations. Gaps of this dimension have never been shown to be covered by endothelium or epithelium. The smallest defects, however, may not represent sites of free communication between vascular and urinary compartments, because cytoplasm of intrinsic glomerular cells have often been noted by TEM to "seal" small GBM discontinuities.[2-7] It is not clear whether small covered defects represent attempts at GBM repair or are simply an earlier stage of the largest disruptions.

The pathogenic sequence required to produce GBM gaps is not currently understood. Previous detailed morphologic studies have implicated a role for immune complexes, inflammatory cells, or thrombosis and necrosis of capillary loops.[2-7] Both immune complexes and significant numbers of inflammatory cells were absent in this biopsy. Capillary loop thrombosis was observed in occasional loops having an intact endothelial lining, while thrombosed loops with disruptions showed necrosis of both podocytes and endothelial cells. This is consistent with capillary loop thrombosis as the initial lesion. Although the mechanism for loop thrombosis still is unknown, thrombosis seems to promote cell necrosis, eventually resulting in GBM disruption.

In summary, the technique of acellular scanning electron microscopy applied to a case of focal-segmental necrotizing glomerulonephritis has revealed GBM defects which precede crescent formation to direct scrutiny by scanning electron microscopy. The most unexpected finding was the large number of defects within an affected focus. Extension of these preliminary observations by application of this technique to additional examples of human necrotizing glomerulonephritis and animal models of crescentic glomerulonephritis may enhance our understanding of the pathogenesis of this destructive process by clarification of its morphologic expression.

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